Validation of Biomedical Testing Methods

Human Whole Blood Pyrogen Test in 96-well Plates Using Fresh or Cryopreserved Blood

Standard Operating Procedure

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JOINT RESEARCH CENTRE
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ECVAM

Standard Operating Procedure

Human Whole Blood Pyrogen Test in 96-well Plates Using Fresh or Cryopreserved Blood

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THIS SOP WAS AMENDED FOR THE CATCH-UP VALIDATION PHASE ONLY. IT DOES THEREFORE ONLY REPLACE THE PREVIOUS VERSION FOR THIS SERIES OF EXPERIMENTS.

1. INTRODUCTION

The whole blood pyrogen test (in vitro pyrogen test IPT) is a two-part assay for the detection of pyrogenic contamination. It involves incubation of the sample with human blood, followed by an enzyme immunoassay for the measurement of IL-1β.
A pyrogen is a substance that causes fever. Bacterial contaminations, which contain exogenous pyrogens, can be deadly. This problem is of great significance for drug safety.
Also, medical devices and biologically produced substances obtained from bacteria and other microorganisms may cause release of endogenous pyrogens (e.g., IL-1β).
Exogenous pyrogens include metabolic substances and cell-wall components of microorganisms. These substances are present during the "normal" course of an infectious disease. Infections by Gram-negative and Gram-positive bacteria are equal in frequency. Both of these bacterial types can activate the release of endogenous pyrogens, which cause fever through the thermoregulatory center in the brain. Although these reactions can occur during the "normal" course of an infectious disease, a deadly shock syndrome can occur in the worst case.
Due to these risks, product safety legislation demands rigorous quality checks for pyrogenic contamination of drugs and devices intended for parenteral use. For example, testing in rabbits for medical end products is required in Germany. Products in development and a few end products are allowed to be controlled by the Limulus assay. The first pyrogen assay, based on human whole blood stimulation by pyrogens, was developed by Hartung et al. (3,4).

2. PURPOSE

This assay simulates *in vitro* the normal human reaction to exogenous pyrogens. A few drops of human blood are mixed with the sample, and exogenous pyrogens in the sample are recognized by immunocompetent cells in the human blood.

These cells release IL-1β, which is measured by an integrated ELISA system.

3. SCOPE / LIMITATIONS

Limit of detection is ≤ 0.25 EEU/ml, not suitable for test samples interfering with blood cytokine release.

4. METHOD OUTLINE

The procedure has two parts:
1) Incubation of the sample with (diluted) human blood.
2) An enzyme immunoassay for the measurement of IL-1β.

Ad 1) Blood incubation
Diluted human whole blood is incubated for 10-24 hours together with saline or RPMI and the sample in a pyrogen-free microtiter plate and aliquots are taken for further examination.

Ad 2) Capture of Endogenous Pyrogens (ELISA procedure)
Samples (aliquots of whole blood stimulation) are distributed into the wells of a microplate which are coated with antibodies specific for IL-1β.

An enzyme-conjugated antibody against IL-1β is added. During a 90-minute incubation, a sandwich complex consisting of two antibodies and the IL-1β is formed. Unbound material is removed by a wash step.

A chromogenic substrate (3,3′,5,5′ -tetramethylbenzidine, TMB) reactive with the enzyme label is added. Color development is terminated by adding a stop solution after 30 minutes. The resulting color, read at 450 nm, is directly related to the IL-1β concentration. Bi-chromatic measurement with a 600-690 nm reference filter is recommended.

5. DEFINITIONS / ABBREVIATIONS

The following abbreviations are used in this work-book.

Ab	antibody
°C	degrees Celsius (Centigrade)
EC	endotoxin control
EEU	endotoxin equivalent unit
ELISA	Enzyme-Linked ImmunoSorbent Assay
EU	endotoxin unit of the international WHO standard
h	hour
HCl	hydrochloric acid
IL	interleukin
LPS	lipopolysaccharide (exogenous pyrogen from Gram-negative bacteria)
LTA	lipoteichoic acid (exogenous pyrogen from Gram-positive bacteria)
µl	microlitre
mg	milligram
min	minute
ml	millilitre
MTP	microtiter plate
MVD	maximum valid dilution
NaCl	sodium chloride, 0,9%
nm	nanometre
NPC	negative product control
PPC	positive product control
OD	optical density
rpm	rounds per minute
RT	room temperature
TMB	3,3´,5,5´-Tetramethylbenzidine

6. MATERIALS

6.1. Materials required and not provided

The components listed below are recommended, but equivalent devices may also be used: it is the users responsibility to validate the equivalence.
For all steps excluding the ELISA procedure sterile and pyrogen-free materials have to be used (e.g. tips, containers, solutions).

6.1.1 Materials for fresh blood incubation

Equipment
·Incubator (37°C + 5% CO_2)
·Multipette or adjustable 20 to 100 µl pipetters
·Multichannel pipettor, 8 or 12 channels
·Vortex mixer
·Laminar flow bench (recommended)

Consumables
·Heparinized tubes for blood sampling(Sarstedt S-MONOVETTE 7.5 ml, 15 IU/ml Li-Heparin)
·Sarstedt multifly needle set, pyrogen-free, for S-Monovette
·Non-pyrogenic 96-well polystyrene tissue culture microtiter plate, Falcon, Cat. No. 353072
·Sterile and pyrogen-free tips 20 µl and 100 µl
·Combitips for multipette, 1.0 ml and 0.5 ml
·Reservoir for saline
·Non-pyrogenic test tubes, preferably 12 or 15 ml centrifuge tubes from greiner bio-one or other qualified materials that can be used for preparing standards and diluting samples

6.1.2 Materials for incubation with cryopreserved blood

Equipment
·Incubator (37°C + 5% CO_2)
·Multipette or adjustable 20 to 100 µl pipetters
·Multichannel pipettor, 8 or 12 channels
·Vortex mixer
·Laminar flow bench (recommended)

Consumables
·Non-pyrogenic 96-well microtiter plate, Falcon, Cat No. 353072
·Sterile and pyrogen-free tips 20 µl and 100 µl
·Combitips for multipette, 1.0 ml and 0.5 ml
·Reservoirs for RPMI and saline
·Non-pyrogenic test tubes, preferably 12 or 15 ml centrifuge tubes from greiner bio-one or other qualified materials that can be used for preparing standards and diluting samples

6.1.3 Materials for ELISA procedure

Equipment
· Multichannel pipettor
· Microplate mixer
· Microplate washer (optional)
· Microplate reader capable of readings at 450 nm (optional reference filter in the range of 600-690 nm)
· A software package for facilitating data generation, analysis, reporting, and quality control

Consumables
· Graduated cylinder and plastic storage container for Buffered Wash Solution
· Tip-Tubs for reagent aspiration with Multichannel pipettor
· non-sterile pipette tips
· non-sterile deionized water

6.2. Materials Supplied in ELISA kit

Components supplied in that kit are *not* interchangeable with other lots of the same components.
IL-1β Ab-coated Microplate: One 96-well polystyrene microplate, packaged in a zip-lock foil bag, with desiccant. The plate consists of twelve strips mounted in a frame. Each strip includes eight anti-IL-1β Ab-coated wells. Additionally, individual strips can be separated from the frame to enable the repackaging and later use of all the wells of a kit. In this case, repackage the strips in the zip-lock foil with the desiccant, reseal the foil airtight and use the strips within 4 weeks. Well positions are indexed by a system of letters and numbers (A through H, 1 through 12) embossed on the left and top edges of the frame. Store refrigerated: stable at 2-8°C until the expiration date marked on the label.
Enzyme-Labeled Antibody : One amber vial containing 21 ml of liquid reagent, ready-to-use. The reagent contains horseradish peroxidase-labeled, affinity-purified, polyclonal anti-IL-1β antibodies, with preservative. Store refrigerated: stable at 2-8°C until the expiration date marked on the label. *Do not freeze.*
Saline: Three plastic vials, each containing pyrogen-free saline. This is intended for the dilution of fresh blood, samples and for reconstitution of the Endotoxin Control. Store refrigerated: stable at 2-8°C until the expiration date marked on the label. Use immediately after opening and discard unused volumes.
RPMI: One plastic vial, containing pyrogen-free RPMI. This is intended for dilution of cryopreserved blood. Store refrigerated: stable at 2-8°C until the expiration date marked on the label. Use immediately after opening and discard unused volumes.
TMB/Substrate Solution: Two amber vials, each containing 11 ml of a buffered reagent, ready-to-use. The reagent contains a hydrogen peroxide substrate and 3,3′,5,5′-tetramethylbenzidine (TMB). Store refrigerated and protected from light: stable at 2-8°C until the expiration date marked on the label. *Do not freeze.*
Buffered Wash Solution Concentrate: One vial containing 100 ml of a concentrated (10X) buffered saline solution, with surfactants and preservative. Using a transfer container, dilute the contents of the vial with **900 ml** distilled or deionized water for a total volume of **1000 ml**.
Store refrigerated: stable at 2-8°C until the expiration date marked on the label.

Stop Solution: One vial containing an acidic solution, for terminating the color reaction. The reagent is supplied ready-to-use. Handle with care, using safety gloves and eye protection. Store refrigerated: stable at 2-8°C until the expiration date marked on the label.

Additionally supplied materials

Endotoxin Control: One vial of an endotoxin control. The control is supplied lyophilized. Before use, reconstitute control vial with pyrogen-free distilled water. Prepare serial dilutions in saline (see 7. Methods). Mix by vortexing. After preparation, the stock solution can be stored (see 7. Methods).
PPC (Positive Product Control): one glass vial containing 1.05 ng/ml Endotoxin. Store at 2-8°C and use according to 7. Methods.

7. METHODS

7.1. Fresh Blood Incubation (Method 7 A)

Blood Collection
Collect blood by venipuncture into heparinized tubes. The blood collection system must be pyrogen-free. The procedure calls for 20 µl of heparinized whole blood per well. The blood can be stored in the collection tube at room temperature (15-28°C) for 4 hours. Incubation of the sample should be started within this time. Prior to use, gently invert the collection tube once or twice. **Do not vortex.**

Note:

1 Blood donors are to describe themselves as in good health and not in need of medication for the last two weeks.
2 Each assay should include the Endotoxin Controls and the saline control in quadruplicate.
3 Use disposable tip pipets to avoid contamination of reagents and samples.
4 During ELISA procedure, the wells should be washed carefully.
5 The test samples should be done in quadruplicate.
6 The contents of the wells must be decanted or aspirated completely before pipetting wash solution.
7 Deviations from the procedure (incubation time/temperature) may cause erroneous results. The ELISA procedure should be run without interruption. Diluted samples should be tested within an hour.

7.2. Blood incubation with cryopreserved blood (Method 7 B, Method 7 C)

Blood frozen according to the Konstanz method has to be stored in the vapour phase of liquid nitrogen
Blood frozen according to the PEI method can be kept at –80°C or in the vapour phase of liquid nitrogen; for longer storage, please transfer the vials into the vapour phase of liquid nitrogen.

Thawing procedure
Take the required number of aliquots out of the vapor phase of liquid nitrogen/the freezer and leave the blood to thaw in the incubator at 37°C for 15 minutes. After this time, dry the condensed water off the vials using a paper cloth. Preferably under a laminar-flow bench, unscrew the vials and pool the blood in a polypropylene centrifuge tube. Gently invert the tube once or twice to achieve complete mixing. **Do not vortex.**

Storage of the substances

- please keep all substances and spikes at 4°C

Spiking of the substances

Part 1)
5 blinded spikes have been sent out by Konstanz
They are bearing a code for
a) the respective drug
b) a random blinding number

- please pipet 500 µl of the respective substance into a test tube
- vortex the respective vial with the blinded spike for about 5 seconds
- add 25 µl of the spike to the substance and vortex for another 5 seconds
- perform the dilutions according to the instructions below

In case of little substance, the amounts may be reduced to 250 µl of substance + 12.5 µl of spike.

Dilution of the substances

- for dilution, please use either 12 ml or 15 ml tubes from greiner bio-one
- each substance has to be vortexed for about 5 seconds immediately before performing Step 3 of the Whole Blood Stimulation.

Substance 1: Glucose 5%
Maximum valid dilution: 1:70; add 50 µl of substance to 3450 µl of saline

Substance 2: EtOH 13%
Maximum valid dilution: 1:35 ; add 100 µl of substance to 3400 µl of saline

Substance 3: MCP
Maximum valid dilution: 1:350; add 10 µl of substance to 3490 µl of saline

Substance 4: Syntocin
Maximum valid dilution: 1:700: add 5 µl of substance to 3495 µl of saline

Substance 5: Binotal
Maximum valid dilution: 1:140; add 25 µl of substance to 3475 µl of saline

Substance 6: Fenistil
Maximum valid dilution: 1:175; add 20 µl of substance to 3480 µl of saline

Substance 7: Sostril
Maximum valid dilution: 1:140; add 25 µl of substance to 3475 µl of saline

Substance 8: Beloc
Maximum valid dilution: 1:140; add 25 µl of substance to 3475 µl of saline

Substance 9: Drug A
Maximum valid dilution: 1:35; add 100 µl of substance to 3400 µl of saline

Substance 10: Drug B
Maximum valid dilution: 1:70; add 50 µl of subsubstance to 3450 µl of saline

Part 2)
(unblinded)

- Positive Product Control (PPC)
dilute the respective substance according to the instructions above
vortex for about 5 seconds
pipet 500 µl of the diluted substance into a pyrogen-free tube
add 25 µl of the unblinded PPC-LPS spike

- Negative Product Control (NPC)
dilute the respective substance according to the instructions above
vortex for about 5 seconds
pipet 500 µl of the diluted substance into a pyrogen-free tube
add 25 µl of saline

Endotoxin dilution for the Dose-Response Curve

IPT assays must include the 0.5 EU/ml + saline control in quadruplicate.

Dissolve the contents of the vial containing O113 provided by NIBSC with 5 ml of pyrogen-free distilled water yielding a stock solution of 2000 EU/ml. After reconstitution of the lyophilisate, vortex the stock solution according to the Certificate of Analysis. Vortex all dilutions prior to use for 5 seconds.

EC = Endotoxin Control, for use in the assay.

Solution	amount added to saline	Volume of saline	Resulting solution
Stock (2000 EU/ml)	100 µl	900 µl	200 EU/ml
200 EU/ml	100 µl	900µl	20 EU/ml
20 EU/ml	100 µl	900 µl	2 EU/ml
2 EU/ml	500 µl	500 µl	1 EU/ml (EC)
1 EU/ml	500 µl	500 µl	0,5 EU/ml (EC)

The stock solution of the Endotoxin Standard may be aliquoted (e.g. 100 µl aliquots) and kept at –20 °C for up to 6 months. Do not store the O113 at –80°C.

Whole Blood Stimulation, fresh blood (Method 7 A)

Perform incubation of blood samples in a microtiter plate.Preferably, use a laminar-flow bench. All consumables and solutions have to be sterile and pyrogen-free.

Step 1: Draw up an incubation plan according to the template below

Step 2: Add **200 µl** saline into each well.

Step 3: Add **20 µl** of Endotoxin Controls and negative saline control or samples in quadruplicate into the respective wells according to the prepared incubation plan.

Step 4: Add **20 µl** of donor blood, mixed by gentle inversion, into each well.

Step 5: Mix the contents of the wells thoroughly by gently aspiring and dispensing them 5 times, using a multichannel pipettor, changing the tips between each row in order to avoid cross-contamination.

Step 6: Put the lid on the plate and place the plate in an incubator at 37°C + 5%CO_2 for 10-24 hours.

Step 7: When transferring the stimulation aliquots onto the ELISA plate, mix the contents of the wells thoroughly by gently aspiring and dispensing them 3 times, using a multichannel pipettor, changing the tips between each row in order to avoid cross-contamination.

The aliquots can be tested immediately by the ELISA System or may be stored at -20°C or –80°C for testing at a later time. After transfer onto the ELISA plate, keep the remaining stimulation aliquots in the incubation plate at 20/-80°C for eventual repetition of the ELISA procedure (see Minimum assay suitability requirements).

Whole Blood Stimulation, cryopreserved blood (Method PEI = Method 7 B)

Perform incubation of blood samples in a microtiter plate. Preferably, use a laminar-flow bench. All consumables and solutions have to be sterile and pyrogen-free.

Step 1: Draw up an incubation plan according to the template below.

Step 2: Add **180 µl** RPMI into each well.

Step 3: Add **20 µl** of Endotoxin Controls and negative saline control or samples in quadruplicate into the respective wells according to the prepared incubation plan.

Step 4: Add **40 µl** of donor blood, mixed by gentle inversion, into each well.

Step 5: Mix the contents of the wells thoroughly by gently aspiring and dispensing them 5 times, using a multichannel pipettor, changing the tips between each row in order to avoid cross-contamination.

Step 6: Put the lid on the plate and place the plate in an incubator at 37°C + 5%CO_2 for 10-24 hours.

Step 7: When transferring the stimulation aliquots onto the ELISA plate, mix the contents of the wells thoroughly by gently aspiring and dispensing them 3 times, using a multichannel pipettor, changing the tips between each row in order to avoid cross-contamination.

The aliquots can be tested immediately by the ELISA System or may be stored at -20°C or –80°C for testing at a later time. After transfer onto the ELISA plate, keep the remaining stimulation aliquots in the incubation plate at 20/-80°C for eventual repetition of the ELISA procedure (see Minimum assay suitability requirements).

Whole Blood Stimulation, cryopreserved blood (Method Konstanz= Method 7 C)

Perform incubation of blood samples in a microtiter plate. Preferably, use a laminar-flow bench. All consumables and solutions have to be sterile and pyrogen-free.

Step 1: Draw up an incubation plan according to the template below

Step 2: Add **200 µl** RPMI into each well.

Step 3: Add **20 µl** of Endotoxin Controls and negative saline control or samples in quadruplicate into the respective wells according to the prepared incubation plan.

Step 4: Add **20 µl** of donor blood, mixed by gentle inversion, into each well.

Step 5: Mix the contents of the wells thoroughly by gently aspiring and dispensing them 5 times, using a multichannel pipettor, changing the tips between each row in order to avoid cross-contamination.

Step 6: Put the lid on the plate and place the plate in an incubator at 37°C + 5% CO_2 for 10-24 hours.

Step 7: Take the plate out of the incubator and freeze it at –20 or –80°C until the contents of the wells are completely frozen. After this, thaw the plate at room temperature or in a water bath at no more than 37°C.

Step 8: When transferring the stimulation aliquots onto the ELISA plate, mix the contents of the wells thoroughly by gently aspiring and dispensing them 3 times, using a multichannel pipettor, changing the tips between each row in order to avoid cross-contamination.

The aliquots can be tested immediately by the ELISA System or may be stored at -20°C or –80°C for testing at a later time. After transfer onto the ELISA plate, keep the remaining stimulation aliquots in the incubation plate at 20/-80°C for eventual repetition of the ELISA procedure (see Minimum assay suitability requirements).

7.2. ELISA Procedure

Remove the ELISA kit from the refrigerator at least 30 minutes before use. All components must be at room temperature (15-28°C). The ELISA is carried out at room temperature.

1 Sample distribution: see Microplate Template below.

A	NPC (A)	NPC (A)	PPC (A)	PPC (A)	PPC (A)	PPC (A)	1 (B)	1 (B)	1 (B)	1 (B)	2 (C)	2 (C)
B	NPC (A)	NPC (A)	1 (A)	1 (A)	1 (A)	1 (A)	2 (B)	2 (B)	2 (B)	2 (B)	2 (C)	2 (C)
C	EC 1,0	EC 1,0	2 (A)	2 (A)	2 (A)	2 (A)	3 (B)	3 (B)	3 (B)	3 (B)	3 (C)	3 (C)
D	EC 1,0	EC 1,0	3 (A)	3 (A)	3 (A)	3 (A)	4 (B)	4 (B)	4 (B)	4 (B)	3 (C)	3 (C)
E	EC 0,5	EC 0,5	4 (A)	4 (A)	4 (A)	4 (A)	5 (B)	5 (B)	5 (B)	5 (B)	4 (C)	4 (C)
F	EC 0,5	EC 0,5	5 (A)	5 (A)	5 (A)	5 (A)	NPC (C)	NPC (C)	NPC (C)	NPC (C)	4 (C)	4 (C)
G	saline	saline	NPC (B)	NPC (B)	NPC (B)	NPC (B)	PPC (C)	PPC (C)	PPC (C)	PPC (C)	5 (C)	5 (C)
H	saline	saline	PPC (B)	PPC (B)	PPC (B)	PPC (B)	1 (C)	1 (C)	1 (C)	1 (C)	5 (C)	5 (C)

A, B, C : e.g. Substances 1, 2, 3
1-5 : blinded spikes 1-5
EC : Endotoxin Control

NPC: negative product control
PPC: positive product control

2 Add **100 µl** Enzyme-Labeled Antibody to every well.

3 Within 10 minutes, pipet **100 µl** of whole blood stimulations of Endotoxin Controls, those of the negative saline control and of the samples into the wells prepared. During transfer, resuspend the contents of the wells of the incubation plate by aspiring and dispensing them 3 times.

Use a disposable-tip micropipet for the samples, changing the tip between each sample and control, to avoid contaminations.

4 Seal the plate with the adhesive foil provided in the kit.

5 Mix for **90 minutes** on a microplate mixer at 350-400 rpm.

6 Decant, then wash. Wash each well 4 times with 250-300 µl Buffered Wash Solution.

If this step is performed manually, remove as much moisture as possible during the decanting by inverting the washed microplate and tapping out the residual washing buffer on blotting paper or a paper towel, being careful not to dislodge the strips from the frame. Perform this step before adding the TMB.

7 Add **200 µl** of TMB/Substrate Solution to every well.

8 Incubate without shaking for **15 minutes** in the dark. Reduce incubation time if necessary (see Minimum assay suitability requirements).

9 Add **50 µl** of Stop Solution to every well.

*Tapping the plate **gently** after the addition of Stop Solution will aid mixing and improve precision. The Stop Solution is acidic.*
Handle carefully, and use safety gloves and eye protection.
10 Read at 450 nm, **within 15 minutes** of adding Stop Solution. Bi-chromatic measurement with a reference wavelength of 600-690 nm is recommended.

MINIMUM ASSAY SUITABILITY REQUIREMENTS

The assay should be considered acceptable only if the following minimum criteria are met:

The mean OD of the 0.5 EU/ml endotoxin control is at 1.6 times the mean OD of the negative saline control or greater.

The mean OD of the PPC is at 1.6 times the mean OD of the NPC or greater.

The mean OD of the PPC has to be in the 50-200 % range of the mean OD of the 0.5 EU/ml endotoxin control.

The mean OD of the negative saline control is at 100 mOD or lower.

If one OD value of the of the 1.0 EU/ml Endotoxin Control is > Max, the ELISA procedure may be repeated, reducing the incubation time (Step 8 of 7.2. ELISA Procedure).

8. HEALTH SAFETY AND ENVIRONMENT

· For *in vitro* use only.
· Do not use reagents beyond their expiration dates.

Bio-Safety
Human blood has to be considered infectious and handled accordingly. When handling nitrogen and the unopened vials of cryopreserved blood, wear protective eyewear. Wear gloves when performing incubations.

Stop Solution and TMB/Substrate Solution
Avoid contact with the Stop Solution, which is acidic. Wear gloves and eye protection. If this reagent comes into contact with skin, wash thoroughly with water and seek medical attention, if necessary. The reagent is corrosive; therefore, the instrument employed to dispense it should be thoroughly cleaned after use. The TMB/Substrate Solution contains peroxide. Since peroxides are strong oxidizing agents, avoid all bodily contact with the TMB/Substrate Solution

9. ANNEX (Pipetting scheme for the whole blood assay)

Part 1a: Whole blood stimulation, fresh blood (all values in µl) (Test 7.1)

well account	Stimulation sample	saline	Endotoxin Control (0.5 – 1.0 EU/ml)	Test sample	Donor blood		
4	Endotoxin Control (0.5 – 1.0 EU/ml)	200	20	-	20	Mix the samples. Incubate overnight at 37°C + 5% CO_2	Mix the samples. Test immediately with the ELISA system or store at -20/-80 °C.
4	Blank (0)	220	-	-	20		
4	Test samples (1-10)	200	-	20	20		

Part 1b: Whole blood stimulation, cryopreserved blood, PEI method (all values in µl) (Test 7.2)

well account	Stimulation sample	RPMI	saline	Endotoxin Control (0.5 – 1.0 EU/ml)	Test sample	Donor blood		
4	Endotoxin Control (0.5 – 1.0 EU/ml)	180	-	20	-	40	Mix the samples. Incubate overnight at 37°C + 5% CO_2	Mix the samples. Test immediately with the ELISA system or store at -20/-80 °C.
4	Blank (0)	180	20	-	-	40		
4	Test samples (1-10)	180	-	-	20	40		

Part 1c: Whole blood stimulation, cryopreserved blood, Konstanz method (all values in µl) (Test 7.3)

well account	Stimulation sample	RPMI	saline	Endotoxin Control (0.5 – 1.0 EU/ml)	Test sample	Donor blood		
4	Endotoxin Control (0.5 – 1.0 EU/ml)	200	-	20	-	20	Mix the samples. Incubate overnight at 37°C + 5% CO_2	Mix the samples. Test immediately with the ELISA system or store at -20/-80 °C.
4	Blank (0)	200	20	-	-	20		
4	Test samples (1-10)	200	-	-	20	20		

Part 2: ELISA procedure (all values in µl)

Supernatants from Stimulation	Enzyme-labeled Antibody		Substrate		Stop solution	
100	100	Incubate 90 min at RT on a plate mixer at 350-400 rpm. Decant.Wash 4 times with 300 µl Buffered Wash Solution	200	Incubate 15 min at RT in a dark place	50	Read at 450 nm (600-690 nm reference wave-length recom-mended)

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